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Changes in Plasma Ghrelin Concentrations in Vitamin A–restricted Japanese Black Steers

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Abstract

The objective of this study was to establish the effect of vitamin A (VA) feeding status on the plasma concentrations of acylated ghrelin, insulin, glucose, and leptin in Japanese Black steers. Eight 14-month-old Japanese Black steers were fattened for 58 weeks. The experimental period was divided into stages 1 (weeks 1–28), 2 (weeks 29–44), and 3 (weeks 45–58). The steers were divided into 3 groups: S (n = 2, VA supplemented), R (n = 3, VA restricted), and RS (n = 3, VA restricted in stages 1 and 2 and supplemented in stage 3). In stage 2, groups R and RS showed lower plasma VA and ghrelin concentrations than those in group S. In stage 3, group R showed lower values for the above concentrations than those in groups S and RS. Group RS showed lower values for the above concentrations and metabolizable energy (ME) intakes in the VA-restricted stage 2 than in the VA-supplemented stage 3. However, VA restriction did not affect the plasma concentrations of insulin, glucose, or leptin. In conclusion, long-term VA restriction decreased the plasma concentrations of VA and acylated ghrelin as well as ME intake in Japanese Black steers. Decreased ghrelin levels may be associated with the decreased feed intake in the VA-restricted cattle.

Key words: fattening, ghrelin, Japanese Black steers, vitamin A restriction

Introduction

In Japan, the market value of a beef carcass greatly depends on the marbling score, which is determined by the degree of marbling of intramuscular fat in the *longissimus thoracis* muscle¹¹⁾, and meat with a high score is expensive²⁰⁾. Several factors, including the breed^{13,25)}, length of the fattening period²¹⁾, type of grain³³⁾, and

amount of feed²⁵⁾, can affect marbling or the intramuscular fat content. Vitamin A (VA) restriction contributes to high-quality beef production by increasing both the amount of marbling^{6,7,19,24)} and the level of monounsaturated fatty acid in meat²⁴⁾. However, VA is an essential factor for several biological processes in mammalian species³⁾. During the fattening of Japanese Black steers, which is known as breed with excellent marbling, VA restriction throughout

the fattening period reduces feed intake and causes intermuscular edema¹⁰⁾. Reduced feed intake is a well-known effect of VA restriction in other breeds of cattle^{8,32)}. However, there have been few studies related to appetite-regulating factors in VA-restricted cattle. There have been previous studies regarding leptin^{10,29)} and insulin²⁹⁾ in VA-restricted steers as well as glucose and insulin-like growth factor I in vitamin A-, D₃-, and E-restricted steers¹⁴⁾. In those studies, VA restriction did not influence these appetite-regulating factors. However, other factors may influence feed intake in VA-restricted cattle.

Here, we focused on another peptide hormone, ghrelin. Ghrelin, which was discovered in the stomachs of rats¹²⁾, is believed to stimulate appetite¹⁵⁾. Furthermore, in cattle, ghrelin-positive cells are present in the abomasum⁹⁾, and ghrelin administration increases the time spent eating and dry matter intake in beef cattle³¹⁾. However, there have been no reports regarding ghrelin in VA-restricted cattle. We hypothesized that VA restriction affects ghrelin concentrations in cattle. The objective of this study was to evaluate the effect of VA restriction on circulating levels of hormones, including ghrelin, and glucose in fattening Japanese Black steers.

Materials and Methods

Animals, management, and treatments

All animals received humane care as outlined in the Guide for the Care and Use of Experimental Animals (Animal Care Committee, NARO Institute of Livestock and Grassland Science).

The experiment included eight half-sibling Japanese Black steers, aged 14 months. Those steers were bought at a livestock market and the experiment was started after the quarantine period and the animals were housed in a pen and mangers with door feeders (Orion Machinery, Suzaka, Japan) equipped with automatic locks that respond to a magnetic device attached to the cattle's collar. This enables the feed intake measurement for each animal in the group housing. Their mean body weight (BW) \pm standard error (SE) was 338.2 ± 13.8 kg. The experiment duration was 58 weeks. The periods from weeks 1–28, weeks 29–44, and weeks 45–58 were defined as stages 1, 2, and 3, respectively. The steers were randomly assigned to three groups for VA treatment.

Group S (n = 2) received weekly oral supplementation of 296.8 IU VA/kg BW (42.4 IU/kg BW/day) throughout the experimental period. Group R (n = 3) was subjected to restricted VA administration, whereby once supplemental VA per 4 weeks was administered only when the plasma VA concentration was near or below 30 IU/dL to prevent deficiency, according to the feeding standard¹⁾. Group RS (n = 3) was subjected to restricted VA administration, as described for group R during stages 1 and 2, but received oral VA supplementation as described for group S during stage 3. The supplement was VA palmitate (2000 IU/g as VA; BASF Vitamins Co., Ltd., Tokyo, Japan). No steers exhibited serious symptoms of VA deficiency as a result of the VA treatment in this experiment.

The feeding conditions were designed according to the Japanese Feeding Standard for Beef Cattle¹⁾ and were typical for beef cattle in Japan. Compositions of the experimental diets are shown in Table 1. The designed concentrate to roughage ratios were 75:25 for stage 1 and 91:9 for stages 2 and 3. Timothy hay was used as roughage in stage 1, and rice straw, which generally contains low levels of the VA precursor β -carotene, was used in stages 2 and 3. The steers were offered enough feed to obtain daily gain (DG) of 1.0 kg in stages 1 and 2 and 0.7 kg in stage 3. The steers were housed in a pen equipped with an individual discriminating door feeder and were fed once daily at 09:00 h. Fresh water was always available.

BW and metabolizable energy (ME) intake

BW was measured before feeding at the beginning

Table 1. Composition of experimental diets

	Stage	
	1	2 and 3
Ingredient (%)		
Timothy hay	25.0	0.0
Rice straw	0.0	9.0
Flaked barley	25.5	42.8
Flaked corn	22.5	31.8
Wheat bran	16.5	10.0
Soybean meal	9.0	5.0
Calcium carbonate	1.0	0.5
Salt	0.5	0.9
Crude Protein (% dry matter) ¹	16.9	10.9
ME (MJ/kg dry matter) ¹	12.3	12.9

¹ Values calculated from Standard Tables of Feed Composition in Japan¹⁶⁾

of the experiment (week 0) and every 2 weeks. ME intake was calculated from the daily recording of dry matter intakes and Standard Tables of Feed Composition in Japan¹⁶⁾. The DG and ME intake values were summarized as means for each stage.

Measurement of plasma VA, hormone, and glucose concentrations

Blood samples were collected from the jugular vein of the cattle every 4 weeks before feeding. Blood samples for hormone and glucose measurements were collected into vacuum blood collection tubes containing EDTA·2Na (Venoject II; Terumo, Tokyo, Japan); 5000 Kallikrein Inhibitor Units of aprotinin solution (Trasylo; Bayer, Leverkusen, Germany) were added to 10 mL of blood, and the sample was then placed on ice. Blood samples were then centrifuged at 4 °C for 20 min at 1000 *g*. Plasma samples for the ghrelin assay were acidified using a 1:10-volume of 1 N HCl to preserve the integrity of acylated ghrelin. Plasma samples for VA concentration measurement were collected into tubes containing heparin (Venoject II), placed on ice, and centrifuged at 4 °C for 20 min at 1000 *g*. All samples were stored at –80 °C until analysis. Plasma VA concentrations were measured through high performance liquid chromatography⁵⁾. Acylated ghrelin concentrations were measured using a ghrelin (active) radioimmunoassay (RIA) kit (Linco Research Inc., St. Charles, MO, USA). Insulin concentrations were measured with an insulin Eiken RIA kit (Eiken Chemical Co. Ltd., Tokyo, Japan). Leptin concentrations were measured using a multi-species leptin RIA kit (Linco Research Inc.). The intra- and inter-assay coefficients of variation (CV) for the ghrelin RIA were 3% and 1%, respectively. The insulin was assayed in a single RIA with the intra-assay CV of 3%. The intra- and inter-assay CVs for the leptin RIA were 3% and 4%, respectively. Plasma glucose concentrations were measured using the Glucose CII Test Wako kit (Wako Pure Chemical Industries, Osaka, Japan).

Statistical analyses

All values are expressed as means and SE unless otherwise stated. The DG and ME intake values and plasma VA, hormone, and glucose concentrations were analyzed using the MIXED procedures (SAS Institute Inc., Cary, NC, USA). The VA treatment, stage, and their interaction

were treated as fixed effects, and each steer was treated as a random effect. For the analysis of the DG and ME intake, “variance components” were specified as the covariance structure in the REPEATED statement; for all other factors, “first-order autoregressive” was specified. If a treatment, stage or interaction was significant at $P < 0.05$, the significance was evaluated via multiple comparisons at an alpha level of 0.05 using the LSMEANS statement and specifying TUKEY for the adjust option.

Results

Changes in BW, DG, and ME intake

Figure 1 shows BW changes throughout the experimental period. The mean BW increased during the 58 weeks from 332.3 kg to 698.8 kg in group S, from 352.4 kg to 684.1 kg in group R, and from 328.0 kg to 653.2 kg in group RS. Stage and interaction had significant effects on the least-squares mean for DG (Table 2). Figure 2 shows the changes in DG during the experimental period. As planned, Group S gained approximately 1 kg/day in stages 1 and 2 and approximately 0.7 kg/day in stage 3. Groups R and RS, however, had lower DG values in stages 2 and 3 than in stage 1. Group S exhibited a significant difference in DG between stages 1 and 3, and between stages 2 and 3. Groups R and RS exhibited a significant difference in DG between stages 1 and 2 and between stages 1 and 3. In stage 2, groups R and RS had significantly lower DG values than group S; however, all groups had similar DG values in stage 3.

Stage and interaction had significant effects on

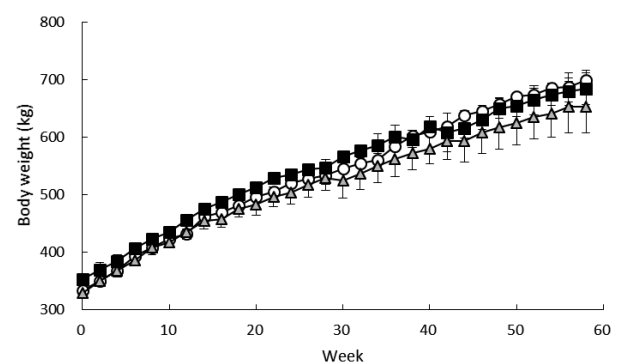


Fig. 1. Changes in mean BW during experiment in VA-supplemented and -restricted steers. Open circles: group S. Closed squares: group R. Gray triangles: group RS.

ME intake (Table 2). Figure 3 shows changes in the mean ME intake. Group S had similar ME intakes in all stages. Group R had lower intakes in stages 2 and 3 than in stage 1, and ME intake was significantly different between all stages. Group RS had a significantly lower ME intake in stage 2 than in stages 1 and 3, and the ME intake in stage 3 was similar to that in stage 1. In stage 2, groups R and RS had lower ME intakes than group S, but the differences between the VA treatment groups were not significant.

Plasma VA concentration

The VA treatment, stage, and interaction significantly affected the VA concentrations of each fattening

stage (Table 2). Figure 4 shows changes in the mean plasma VA concentrations. Group S had concentrations exceeding 80 IU/dL in all stages. Group R had significantly lower concentrations in stages 2 and 3 than in stage 1. Group RS had significantly lower concentrations in stage 2 than in stages 1 and 3. In stage 2, groups R and RS had significantly lower concentrations than those in group S. In stage 3, group R had significantly lower concentrations than those in groups S and RS.

Plasma ghrelin concentrations

The VA treatment and interaction had significant effects on plasma ghrelin concentrations, whereas there

Table 2. Least squares means with standard errors and *P* values in the model for DG, ME intake, plasma vitamin A, glucose and hormone concentrations in VA-supplemented and –restricted steers.

Item	Treatment ¹			Stage ²			<i>P</i> -value		
	Group S	Group R	Group RS	1	2	3	Treatment	Stage	Interaction
DG (kg/day)	0.88±0.08	0.69±0.07	0.75±0.07	1.01±0.05 ^a	0.71±0.05 ^b	0.61±0.05 ^b	0.31	<.001	0.008
ME intake (MJ/day)	102.6±2.8	96.5±2.3	96.6±2.3	104.7±1.6 ^a	92.3±1.6 ^c	98.7±1.6 ^b	0.26	<.001	0.015
Vitamin A (IU/dL)	144.8±13.7 ^a	63.3±11.2 ^b	85.4±11.2 ^b	114.1±7.4 ^a	74.9±8.1 ^b	104.5±8.6 ^a	0.013	<.001	<.001
Ghrelin (pg/mL)	106.5±7.1 ^a	63.3±5.9 ^b	70.6±5.8 ^b	78.7±4.6	71.0±6.4	90.7±7.2	<.001	0.12	0.003
Insulin (μU/mL)	27.0±5.2	22.0±4.2	24.3±4.2	17.0±2.8 ^b	29.4±3.3 ^a	26.9±3.6 ^a	0.77	<.001	0.64
Glucose (mg/dL)	58.0±1.3	55.5±1.1	57.5±1.1	60.0±0.8 ^a	55.1±1.0 ^b	56.0±1.1 ^b	0.34	<.001	0.16
Leptin (ng/mL HE)	8.6±1.3	8.1±1.1	7.3±1.1	6.5±0.7 ^b	8.5±0.7 ^a	8.9±0.8 ^a	0.75	<.001	0.41

^{a-c} Means with different superscripts differ between treatments or stages (*P* < 0.05).

¹ Treatments: R group = Vitamin A feeding restricted; S group = Vitamin A supplemented; RS group = Vitamin A feeding restricted during stages 1 and 2 and supplemented during stage 3

² Stage: 1 = from week 1 to 28; 2 = from week 29 to 44; 3 = from week 45 to 58

DG: Daily gain

ME: Metabolizable energy

HE: Human equivalent

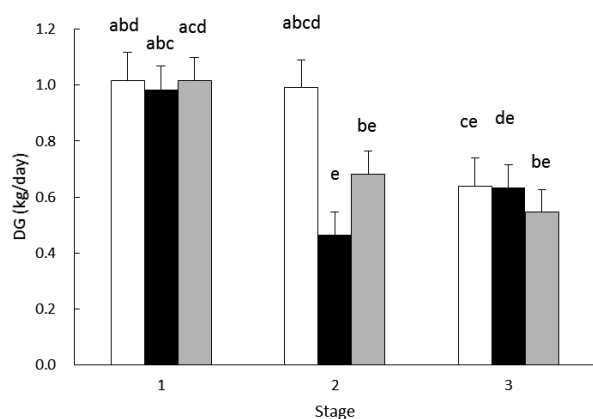


Fig. 2. DG values of each stages in VA-supplemented and -restricted steers (least-squares mean ± SE). Open columns: group S. Closed columns: group R. Gray columns: group RS. ^{a-c} Means with different letters differ significantly (*P* < 0.05).

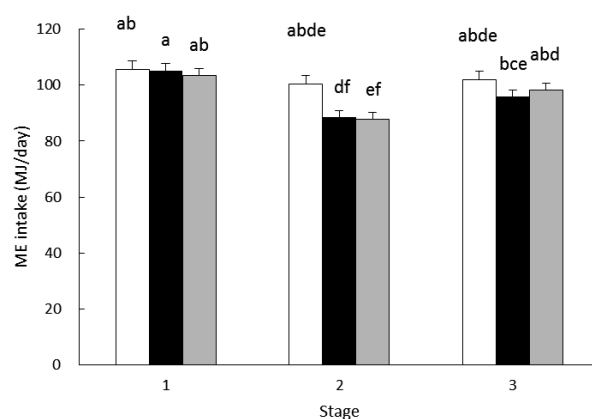


Fig. 3. ME intake of each stages in VA-supplemented and -restricted steers (least-squares mean ± SE). Open columns: group S. Closed columns: group R. Gray columns: group RS. ^{a-f} Means with different letters differ significantly (*P* < 0.05).

was no significant effect of stage (Table 2). Figure 5 shows changes in the mean ghrelin concentrations. Group R had lower concentrations in stages 2 and 3 than in stage 1, but these differences were not significant. Group RS had a lower concentration in stage 2 than in stages 1 and 3, and ghrelin concentrations were significantly different between stages 2 and 3. In stage 2, groups R and RS had significantly lower concentrations than those in group S. In stage 3, group R had lower concentrations than those in groups S and RS, and groups R and S showed difference trend ($P = 0.053$).

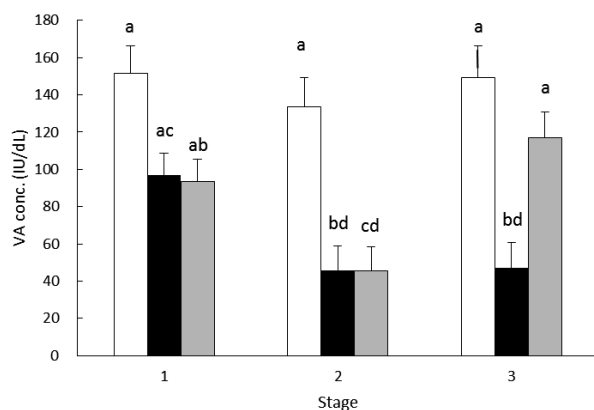


Fig. 4. Changes in VA concentrations in VA-supplemented and -restricted steers during experiment (least-squares mean \pm SE).

Open columns: group S. Closed columns: group R. Gray columns: group RS. ^{a-d} Means with different letters differ significantly ($P < 0.05$).

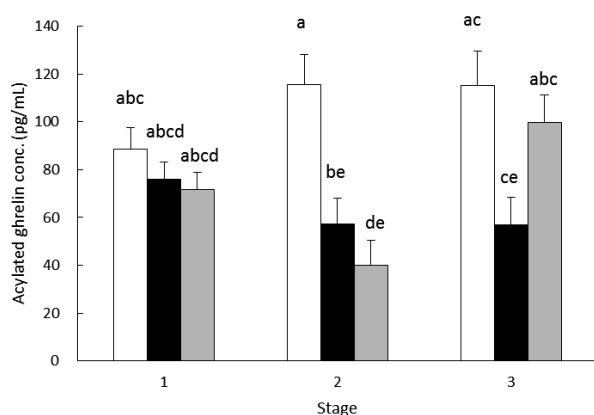


Fig. 5. Changes in plasma ghrelin concentrations in VA-supplemented and -restricted steers during experiment (least-squares mean \pm SE).

Open columns: group S. Closed columns: group R. Gray columns: group RS. ^{a-e} Means with different letters differ significantly ($P < 0.05$).

Plasma insulin, glucose, and leptin concentrations

Stage had significant effects on the plasma insulin, glucose, and leptin concentrations, but there were no significant effects of the VA treatment or interaction (Table 2). The insulin and leptin concentrations were significantly higher in stages 2 and 3 than in stage 1. The glucose concentrations were significantly higher in stage 1 than in stages 2 and 3.

Discussion

In this study, the increases in BW reflected the normal growth of steers. The VA treatments affected both the plasma VA and ghrelin concentrations. The changes in these concentrations were similar in three respects. First, in group RS, the concentrations of both factors were lower in stage 2 than in stages 1 and 3. In this group, the higher concentrations in stage 3 than in stage 2 suggested that, for these factors, the effect of VA feeding status was greater than the effect of feed composition. Second, in stage 2, the plasma VA and ghrelin concentrations were both lower in groups R and RS than in group S. Finally, in stage 3, the concentrations of both factors were lower in group R than in groups S and RS. Whereas, intravenous short chain fatty acid injection decrease blood ghrelin concentration in wethers ⁴⁾. In our study, VA restriction affected the value of ME intakes (Figure 3). It suggests that VA restriction caused the difference in the feed intake, altered the rumen fermentation and short chain fatty acid production, and consequently, decreased the blood ghrelin concentration. However, rumen total short chain fatty acid concentration did not differ between the high concentration and high roughage diet in Holstein steers ¹⁸⁾. In our study, differences in feed intake between the high concentration and high roughage diet were not extreme. It suggests that difference in rumen fermentation was not large between the experimental groups before feeding. Blood short chain fatty acids are considered as transited from rumen. Thus we presume that VA restriction does not largely alter the blood short chain fatty acid concentration before feeding, and that the blood short chain fatty acid did not largely affect the blood ghrelin concentrations. We therefore conclude that in this experiment, VA restriction decreased the ghrelin concentrations in steers and that VA supplementation to VA-restricted steers increased the ghrelin concentrations.

This is the first report to show a decrease in plasma ghrelin concentrations in VA-restricted animals. Our results demonstrate a possible relationship between VA restriction and plasma ghrelin concentrations in cattle. Several factors affect the plasma ghrelin concentrations in cattle, including feed restriction²⁾, negative energy balance³⁰⁾, and intravenous glucose administration²²⁾. However, those factors cannot explain our observed changes in ghrelin concentration following VA restriction. Therefore, other factors, which have not been considered to be related with ghrelin, might be involved. One possible mechanism is the involvement of the nuclear ligand function of VA. VA restriction might reduce ghrelin mRNA expression because VA acts as a nuclear ligand²³⁾ and can regulate mRNA expression of several genes. However, ghrelin is not known as target of nuclear receptors involved in VA. Further studies are required to understand the mechanism that underlies this VA restriction-mediated effect on ghrelin.

In contrast, VA restriction did not affect plasma concentrations of leptin, insulin, or glucose. This result agrees with those from the previous studies^{10,14,28,29)}, indicating that VA restriction, the degree of which did not cause VA deficiency, probably did not reduce the plasma concentrations of these factors in steers. VA restriction was also found to influence ME intake. Groups R and RS exhibited lower ME intakes during VA-restricted stages. A decrease in feed intake is considered as a sign of VA deficiency in cattle^{1,17)}. VA supplementation increased the dry matter intake in a dose-dependent manner in bulls that were fed a low- β -carotene diet³²⁾. Moreover, Japanese Black steers exhibited greatly reduced feed intake at serum retinol, which is the main circulating type of VA, concentrations below 20 IU/dL¹⁰⁾. Our results are consistent with these findings. However, the underlying mechanisms remain unknown. In the present study, similar changes were observed in ghrelin concentrations and ME intakes. In particular, group RS had both lower ghrelin concentrations and lower ME intakes during the VA-restricted stage 2 than during the VA-supplemented stage 3. This similarity suggests an association between plasma ghrelin concentrations and feed intake in VA-restricted steers. Ghrelin is considered to be an appetite stimulator in rodents and humans. In cattle, ghrelin administration stimulates eating behavior³¹⁾. Sheep, which are fed multiple times a day, exhibit a peak serum ghrelin concentration

before feeding, which quickly decreases after feeding^{26,27)}. Moreover, feed-restricted cattle have higher ghrelin concentrations³⁰⁾. These results also suggest an association between eating behaviors and ghrelin concentrations in ruminants. However, VA-restricted cattle exhibit various symptoms¹⁷⁾, suggesting that VA restriction induces several physiological responses. Further studies are required to determine the mechanistic relationship between the feed intake and VA restriction status.

In conclusion, the present study showed that long-term VA-restricted feeding decreased the plasma VA and acylated ghrelin concentrations as well as ME intake in Japanese Black steers. Providing VA supplementation to VA-restricted steers increased the concentrations of both plasma VA and acylated ghrelin as well as ME intake. However, VA restriction did not affect the plasma concentrations of insulin, glucose, or leptin. Decreased plasma ghrelin concentration may reduce the feed intake in VA-restricted cattle.

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ビタミン A 給与制限下の黒毛和種去勢牛における血漿中グレリン濃度の推移

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摘 要

本研究は黒毛和種去勢牛へのビタミン A (VA) 給与制限が血漿中グレリン, インスリン, グルコースおよびレプチン濃度に与える影響を明らかにすることを目的とする。14 ヶ月齢黒毛和種去勢牛 ($n = 8$) を 58 週間肥育した。試験期間はステージ 1 (1 週目より 28 週目まで), 2 (29 週目より 44 週目まで), 3 (45 週目より 58 週目まで) に区切った。試験牛は 3 区に群分けした: S 区 ($n = 2$, VA を給与), R 区 ($n = 3$, VA 給与を制限), および RS 区 (ステージ 1 および 2 は VA 給与を制限, ステージ 3 は給与)。ステージ 2 において, R 区および RS 区は S 区よりも血漿中 VA およびグレリン濃度が低かった。ステージ 3 において, R 区は他の 2 区よりもこれらの値は少なかった。RS 区においては, これらの値および代謝エネルギー (ME) 摂取量は VA を給与されていたステージ 3 よりも VA 給与を制限されていたステージ 2 の方が低かった。一方, VA 給与制限は血漿中インスリン, グルコースおよびレプチン濃度には影響を与えなかった。以上より, 黒毛和種去勢牛に対する長期の VA 給与制限は血漿中 VA 濃度, グレリン濃度および ME 摂取量を減少させると考えられる。VA 給与を制限されたウシにおいて飼料摂取量が減少することと血中グレリン濃度低下には関係がある可能性がある。

キーワード: 肥育牛, グレリン, 黒毛和種, ビタミン A 給与制限